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AN IMPROVED PCR-BASED METHOD FOR GENDER IDENTIFICATION IN BIRDS

WARREN C. CONWAY, JEFFREY K. WICKLIFFE, FEDERICO G. HOFFMANN, ROBERT J. BAKER, AND LOREN M. SMITH

ABSTRACT

Molecular sexing techniques permit unambiguous gender identification in non-ratite birds using PCR and agarose gel electrophoresis. If PCR products are not sufficiently amplified or when length differences between CHD-Z and CHD-W gametologs cannot be visualized using agarose, these techniques fail. We improved and applied a molecular sexing technique using a fluorescently-labeled primer during PCR, followed by capillary electrophoresis and laser excitation to determine gender in 335 Snowy Plover (*Charadrius alexandrinus*) adults and chicks. Males showed a single peak at 368-369 bp (CHD-Z) and females showed two peaks (368-369 bp; CHD-Z) and (382-383 bp; CHD-W). Gender assignment was successful, regardless of PCR product intensity on agarose gels. We found intron length between the Z and W to be no more than 15 bp; too small a difference to be visualized using agarose electrophoresis. We resolved these problems and suggest that this improved technique be used for investigations of avian gender identification.

INTRODUCTION

Male and female hatchling or fledgling birds often appear morphologically indistinguishable and this is also true of adults of many bird species. Nonetheless, proper gender assignment is important for population modeling, particularly for endangered species (Lens et al. 1998) and for examining sex ratio adjustment and sexual selection hypotheses (Bradbury et al. 1997; Pagliani et al. 1999; Questiau et al. 2000). Consequently, with the development of molecular gender identification techniques (Longmire et al. 1993; Griffiths et al. 1996; Kahn et al. 1998; Griffiths et al. 1998; Fridolfsson and Ellegren 1999; Questiau et al. 2000), previously indistinguishable individuals may be unequivocally assigned gender.

Griffiths et al. (1996 and 1998) used a PCR-based procedure to amplify an intron from the CHD-Z and CHD-W genes, followed by agarose gel electrophoresis. This gene is comprised of two gametologs located on the Z and W avian sex chromosomes, where introns of the two gametologs differ in length, but are flanked by highly conserved nucleotide sequence.

Therefore, gametologs are distinguished based upon molecular length using electrophoretic separation. This technique has been successful because of (1) the highly conserved nature of portions of this gene in non-ratites (Kahn et al. 1998; Fridolfsson and Ellegren 1999) and (2) the ability to distinguish CHD-Z and CHD-W gametologs using agarose electrophoresis (Griffiths et al. 1998; Pagliani et al. 1999). However, this technique fails when PCR products cannot be detected on agarose gels (Pagliani et al. 1999; this study) and/or when length differences between CHD-Z and CHD-W gametologs are too small.

In the present study, we modified an existing technique using the CHD (Chromo-Helicase-DNA binding) gene to assign gender in Snowy Plover (*Charadrius alexandrinus*) adults and chicks. Snowy Plover chicks do not exhibit sexual size dimorphism nor can gender be reliably assigned at hatching (Conway 2001). Although adults exhibit morphological and plumage sexual dimorphism, a second adult male phenotype, which resembles the female phenotype, occurs in this population (Conway 2001). As such, inferences regarding sex ratios or population structure using field gender assignments based upon plumage may be severely compromised (Conway 2001). We developed and applied a fluorescent PCR-based gender identification technique using capillary electrophoresis and laser excitation to assign gender in Snowy Plovers.

METHODS

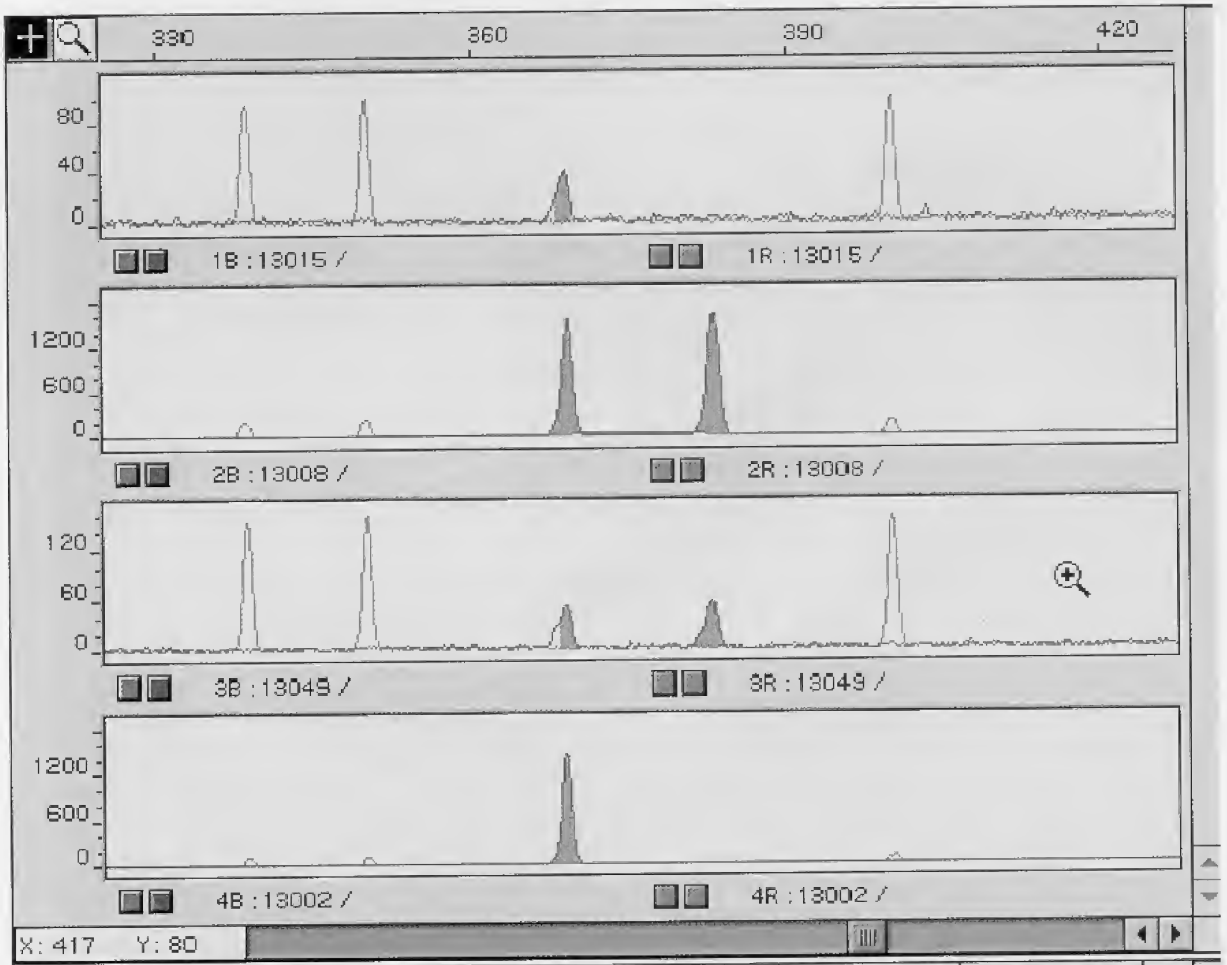
We removed a 200 µl blood sample from adult and hatchling Snowy Plovers captured between March and August 1999 and 2000 in saline lakes in the Southern High Plains of Texas. Hatchlings were captured by hand on the nest, whereas adults were captured with mist nets or via nest traps while incubating (Conway and Smith 2000). We removed blood samples from adults by brachial veinopuncture, whereas blood samples were removed from chicks by puncturing the vein at the tibia-knee joint. Blood was placed into 5 ml of lysis buffer and DNA extractions were performed using standard phenol:chloroform purification (Longmire et al. 1997).

We performed polymerase chain reactions (PCR) in a Perkin-Elmer DNA thermal cycler, using 150 ng of genomic DNA in a 19.6 µl reaction. We used 2.5 µl each of 5 µM CHD primers anchored in conserved flanking sequence (Griffiths et al. 1996) (Integrated DNA Technologies, Inc.; Coralville, IA) P2 [5'-TCT GCA TCG CTA AAT CCT TT-3'] and P8-6FAM (fluorescently labeled) [5'-CTC CCA AGG ATG AGR AAY TG-3'] for the PCR. Each reaction used 9 µl ddH_2O, 2 µl 10X *Taq* buffer (Promega Corp.; Madison, WI), 1.6 µl of 5 mM dNTP's (Applied Biosystems Inc. (ABI); Foster City, CA), 1 µl of 25 mM $MgCl_2$ (Promega Corp.; Madison, WI), and 1U *Taq* DNA polymerase (Promega Corp.; Madison, WI) overlain with light mineral oil. Cycling parameters consisted of an initial denaturation at 95° C for 2 min., followed by 36 cycles with the following thermal profile: denaturation at 95° C for 1 min., primer annealing at 45° C for 1 min., extension at 72° C for 1 min., with a final extension at 72° C for 2 min. A 5 µl sample of each PCR product was also electrophoresed through a (3%) agarose gel in ethidium bromide at 31 V/cm for 2.5-3 h and visualized using a Stratagene Eagle Eye Transilluminator (Stratagene; LaJolla, CA).

We mixed unpurified fluorescent PCR products (1 µl) with 2 µl of an internal size standard, TAMRA 500 (ABI), denatured at 95°C for 5 min. in 12 µl deionized formamide, and immediately snap chilled. Samples were subjected to capillary electrophoresis for 50 min. through a 61 cm capillary filled with POP4 (Performance Optimized Polymer 4, ABI) with a plate temperature of 60°C. Electrophoresis, laser excitation, and analyses were carried out on an ABI Prism® 310 Genetic Analyzer (ABI) using the GeneScan® ver. 2.1 software (ABI).

RESULTS

A total of 335 Snowy Plovers were assigned gender and size peaks were detected at 368-369 bp and 382-383 bp (Fig. 1). Males showed a single peak at 368-369 (CHD-Z) whereas females showed two peaks (368-369 [CHD-Z]; and 382-383 [CHD-W]). Intron length difference between the Z and W gene in this species was determined to be no more than 15 base pairs (Fig. 1). The small difference in length between gametologs cannot be easily and reliably resolved using conventional agarose electrophoresis as an adequate



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
1B, 2	42.80	369.12	41	668	11672
2B, 2	43.95	369.29	1547	28561	11986
2B, 3	44.79	383.08	1614	30422	12215
3B, 3	40.18	368.96	55	789	10956
3B, 4	40.93	382.90	60	1130	11162
4B, 2	41.96	368.92	1457	22752	11443

1. Female snowy plovers are 8021-13008 and 8021-13049 (USFWS band num.).
2. Male snowy plovers are 8021-13015 and 8021-13002 (USFWS band num.).
3. Note the scale for peak height on the Y-axes for all four chromatograms.
4. Empty peaks are TAMRA 500 standard.

Figure 1. Chromatograms of 2 female[1] and 2 male[2] snowy plovers (*Charadrius alexandrinus*) showing the CHD-Z and CHD-W gametologs generated with an ABI Prism 310® Genetic Analyzer using GeneScan® ver. 2.1 (ABI)[3,4].

separation medium to distinguish gametologs (Fig. 2). However, using a short chain polyacrylamide polymer, fluorescent excitation, and capillary electrophoresis, this 15 base pair difference was easily discerned. There were also instances in which PCR products were not sufficiently amplified for visualization on 3% agarose gels, but signal amplification and subsequent gender identification was possible using this new method (Figs. 1, 2).

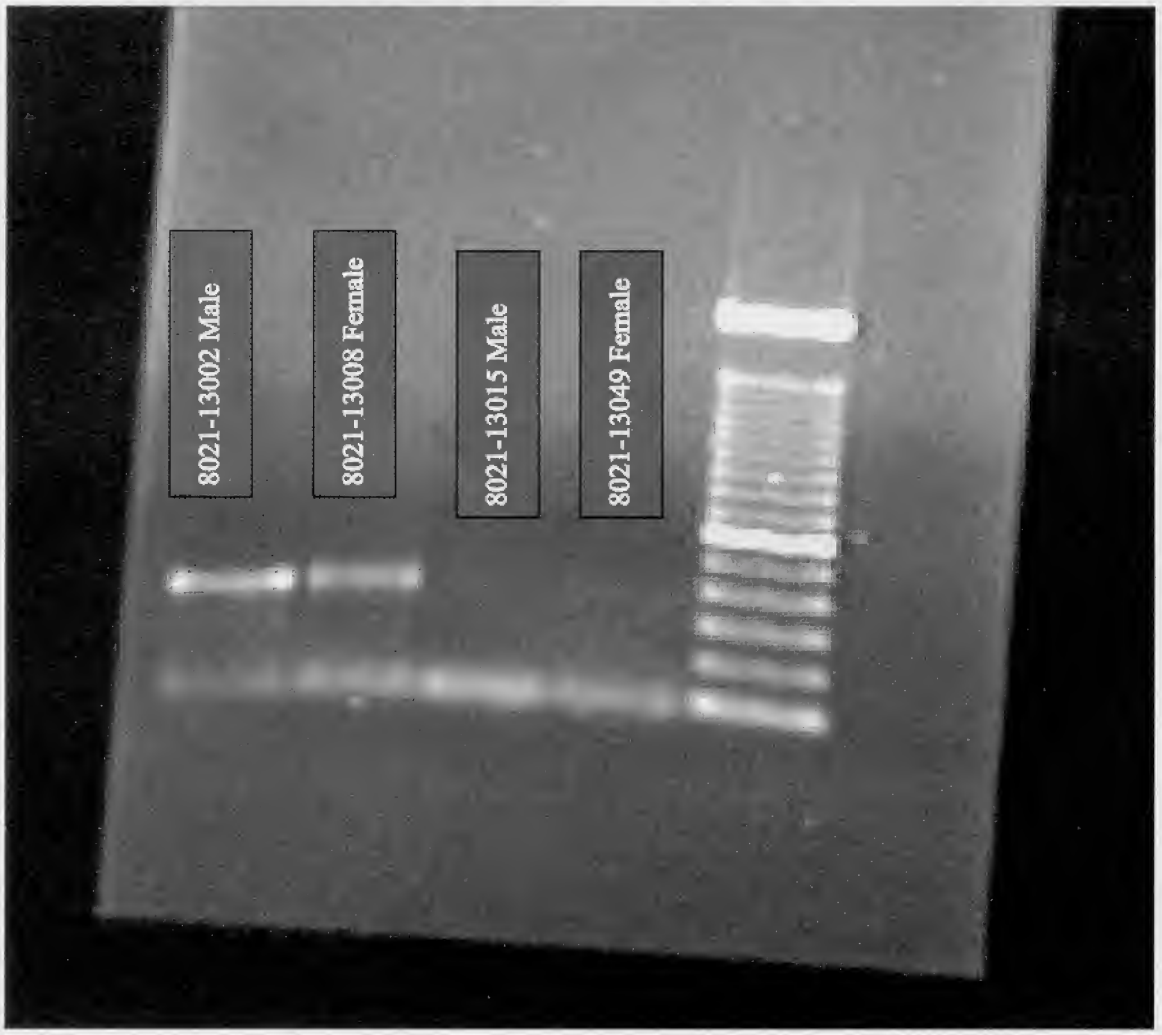


Figure 2. PCR products of snowy plover CHD genes amplified with the primers P2 and P8 (fluorescently labeled). Notice the lack of separation between the CHD-Z and CHD-W gametologs in lanes 1 and 2 and the absence of PCR products in lanes 3 and 4. Both problems were resolved using capillary electrophoresis and laser excitation (please refer to Figure 1).

DISCUSSION

The use of the sex-linked CHD gene in non-ratites has been shown to be relatively universal for assigning gender in birds (Ellegren and Sheldon 1997; Fridolfsson and Ellegren 1999). However, using agarose gel electrophoresis to discriminate between males and females may sometimes be difficult. Our results contrast with other studies (Griffiths et al. 1996; Fridolfsson and Ellegren 1999; Pagliani et al. 1999) which demonstrated that birds from many orders can be successfully assigned gender using only agarose electrophoresis. However, these techniques are cost-effective approaches to determining gender, particu-

larly when distance(s) between gametologs are large enough to visualize after electrophoresis.

The utility of capillary electrophoresis and laser excitation is threefold. First, unless the CHD-Z and CHD-W introns are essentially equal in length, successful gender assignments may be achieved. For example, intron length difference between the Z and W gene is no more than 15 base pairs in Snowy Plovers (Fig. 1), much smaller than the 210-285 base pair difference reported for several bird species in Kahn et al. (1998). However, gender was still easily resolved in this study, despite the small intron length difference. Second, if PCR products are visible, (and in many cases when they are not) this approach is still successful. Finally, this approach is consistent (i.e., quantifiable fragment length) and repeatable, and removes possible subjectivity (i.e., using an internal size standard) while determining fragment lengths.

Further, no enzymatic digestions are required, effectively eliminating time-consuming procedures. Although early investigations of avian molecular gender identification utilized Southern blots and DNA fingerprinting techniques, these approaches are cumbersome (Longmire et al. 1993). Also, these approaches often rely upon null amplifications to determine gender in males, which may arise simply from technical error rather than gender specific non-amplification.

We suggest that this PCR-based technique using capillary electrophoresis and laser excitation be incorporated into investigations of gender identification in birds. This technique may be particularly useful for species in which sexes (i.e., gametologs) cannot be discriminated using agarose gels, or for critical samples in which amplification is not possible. The ease, accuracy, and repeatability of this methodology makes it attractive over previously described techniques, but if such hardware is not available, then this technique will be a cumbersome and expensive proposition. Consequently, if standard agarose electrophoresis is suitable, then previous techniques are still useful. However, if such hardware is available, this technique is relatively inexpensive, highly reproducible, and extremely accurate.

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Addresses of authors:

Warren C. Conway

Wildlife and Fisheries Management Institute
Department of Range, Wildlife, and Fisheries Management
Texas Tech University
Lubbock, Texas, TX 79409-2125

Current Address:

Arthur Temple College of Forestry
Stephen F. Austin State University
Nacogdoches, TX 75962-6109
e-mail: wconway@sfasu.edu

Jeffrey K. Wickliffe

Department of Biological Sciences
Texas Tech University
Lubbock, TX 79409-3191

Current Address:

Department of Preventive Medicine and Community Health
Division of Environmental Toxicology
University of Texas Medical Branch
Galveston, TX 77555-1110
e-mail: jkwickli@utmb.edu

Federico G. Hoffmann

Department of Biological Sciences
Texas Tech University
Lubbock, TX 79409-3191

Current Address:

School of Biological Sciences
University of Nebraska
Lincoln, NE 68588-0118
e-mail: fhoffman@biocomp.unl.edu

Robert J. Baker

Department of Biological Sciences and Museum
Texas Tech University
Lubbock, TX 79409-3191
e-mail: rjbaker@ttu.edu

Loren M. Smith

Wildlife and Fisheries Management Institute
Department of Range, Wildlife, and Fisheries Management
Texas Tech University
Lubbock, TX 79409-2125
e-mail: l.m.smith@ttu.edu

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